ELECTROPHORETIC ISOENZYME PATTERNS OF SOME YEAST STRAINS USED IN WINE INDUSTRY

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ABSTRACT - 12 strains of *Saccharomyces cerevisiae* sensu Yarrow (1984) were studied by gel electrophoresis for 11 isofunctional enzymes. These enzymes included three oxidoreductases (alcohol deshydrogenase, glucose-6-phosphate dèshydrogenase, malate deshydrogenase), seven hydrolases (alkaline and acid phosphatases, and various esterases - α and β naphtyl acetate, β naphtyl propionate, α naphtyl butyrate, indoxyl acetate) and one lyase (aldolase). Polymorphism was evident for most of the enzymes tested, and each strain had a unique pattern when all enzymes were considered. The numerical analysis of the electrophoretic patterns supported the conclusion that all strains were physiological variants of a single taxon. Enzyme electrophoresis was confirmed to be a useful criteria in clarifying taxonomic relationship in yeasts.

RÉSUMÉ - Le comportement enzymatique de 12 souches de *Saccharomyces cerevisiae* sensu Yarrow (1984) utilisées dans l'industrie du vin a été analysé par électrophorèse en gel de polyacrylamide. L'investigation a porté sur 11 enzymes différentes: trois oxydo-réductases (alcool déshydrogénase, glucose-6-phosphate déshydrogénase, malate déshydrogénase), sept hydrolases (phosphatases acide et alcaline, et diverses estérases - α et β naphtyl acétate, β naphtyl propionate, α naphtyl butyrate, indoxyl acétate) et une lyase (aldolase). Le polymorphisme est évident pour la plupart des enzymes recherchées et un profil caractéristique peut être rapporté à chaque souche. L'analyse numérique des profils enzymatiques permet de conclure que toutes les souches étudiées sont en fait des variants physiologiques d'un seul et même taxon. Cette étude confirme la valeur chimiotaxonomique du critère électrophorétique pour la classification des levures.

KEY WORDS : Isoenzymes, *Saccharomyces cerevisiae*, taxonomy, yeasts.

INTRODUCTION

Commercially produced strains of yeast are being widely utilize in the wine industry. Although many factors are involved to determine the qualities of alcoholic beverages, the yeast contribute significantly to the development of their flavor or aroma as well as their organic composition.

Consequently, seeding with dry yeasts, made it necessary to assess more accurately the characterization of these new selected microorganisms. As most of the strains belong to the *Saccharomyces cerevisiae* group, conventional criteria such as morphological and physiological tests commonly employed in yeast taxonomy are not always reliable.

More advanced techniques including serology (Tsuchiya et al., 1965, 1974; Montrocher, 1982), DNA base composition (Nakase & Komagata, 1971; Poncet & Fiol, 1972), proton magnetic resonance spectra of the mannans components (Spencer & Gorin, 1969), co-enzyme Q system (Yamada et al., 1976, 1977), cytochrome spectra (Montrocher & Claisse, 1987) have been applied successfully for yeast characterization.

More recently, electrophoresis of total soluble cell proteins (Van Vuuren & Van Der Meer, 1987; Degré et al., 1989) or macromolecules excreted (Bouix & Leveau, 1983) have been used to distinguish between yeast strains, as well as chromosome electrophoresis (Lalande et al., 1987; Degré et al., 1989; Kaneko & Banno, 1991) or long-chain fatty acid composition (Cottrell et al., 1986; Trédoux et al., 1987; Viljoen et al., 1988).

Comparisons of nucleic acids such as nuclear DNA/DNA reassociation (Meyer & Phaff, 1972; Price et al., 1978; Vaughan Martini & Kurtzman, 1985; Vaughan Martini & Martini, 1987; Kurtzman, 1989a; Kaneko & Banno, 1991) as well as DNA fingerprinting (Pedersen, 1986; Degré et al., 1989), fragment patterns of mitochondrial DNA (Mac Arthur & Clark-Walker, 1983; Dubourdieu et al., 1987) or sequences of 18S and 25S RNA molecule (Lane et al., 1985; Kurtzman, 1989b; Kurtzman & Robnett, 1988, 1991) provide insight into the yeast taxonomy.

Electrophoretic comparison of isoenzymes is a current approach of molecular taxonomy. Such analysis have been applied in various fields: bacteria (Faure-Raynaud et al., 1990), fungi (Chen et al., 1991) and yeasts (Sidenberg & Lachance, 1983; Yamazaki & Komagata, 1981, 1982, 1983; Yamazaki et al., 1983; Yamazaki & Goto, 1985; Yamada & Nagakawa, 1988).

In the present paper, twelve commercial strains of *Saccharomyces cerevisiae*, from various origin and commonly used in wine industry were investigated for electrophoretic enzymes comparison.

MATERIALS and METHODS

1. Microorganisms studied:

Twelve yeast strains were investigated. They all belonged to the species *Saccharomyces cerevisiae* (sensu Yarrow, 1984). They were obtained from Dr. Cuinier (Institut Technique du Vin, Tours, France). The strains, their sources and origin are listed in Table 1.

2. Preparation of free-cell extracts:

The strains were grown aerobically on GYP liquid medium containing glucose $5g.l^{-1}$, yeast extract $2g.l.^{-1}$ and bacto-peptone $2g.l.^{-1}$, for 24 hr on a rotary shaking table, at 25°C.

The cells were harvested by centrifugation at 10,000g (4°C) for 15 mn, and the pellets were washed three times in distillated water.

Cells were disrupted with 0.5 mm glass beads in a Braun homogeneizer for 3mn in Tris-HCl 0.06M pH 6.8 with chilling in ice.

The extracts were centrifugated at 10,000g for 15 mn at 4°C and the supernatants centrifuged again for 2 hrs using the Ti rotor of a Beckman ultracentrifuge at 100,000g. The enzyme preparations were stored at -20°C until analysis.

Table 1: Strains of *Saccharomyces cerevisiae* studied.
Tableau 1: Souches de *Saccharomyces cerevisiae* étudiées.

STRAINS TESTED	SOURCE	TRADE MARK
Strain A	I.T.V *, L-12	Joc-Lalvin
Strain B	I.T.V., L-105	Lalvin
Strain C	I.T.V., L-1090	Lalvin
Strain D	I.T.V., L-311	Fermivin
Strain E	I.T.V., L-395	Fermichamp
Strain F	I.T.V., L 434	Levuline
Strain G	I.T.V., L-448	Levuline
Strain H	I.T.V., L-1077	SB-1
Strain I	I.T.V., L-2056	L-2056
Strain J	I.T.V., L-2266	L-2266
Strain K	I.T.V., L-28	L-28
Strain L	I.T.V., L-905	L-905

* I.T.V.= Institut Technique du Vin, Tours, France.

Protein rate was determinated by the Bradford method (1976), with BSA as standard.

3. Enzyme systems tested:

Eleven enzymes were examinated: aldolase (ALD; EC 41213), alcohol deshydrogenase (ADH; EC 1111), glucose-6-phosphate deshydrogenase (G6PD; EC 11149); malate deshydrogenase (MDH; EC 11137), acid phosphatase (ACP; EC 3132), alkaline phosphatase (AKP; EC 3131) and esterases (EC 3111) α- naphtyl acetate (EST 1), indoxyl acetate (EST 2), β- naphtyl acetate (EST 3), β- naphtyl propionate (EST 4) and α- naphtyl butyrate (EST 5).

All first six enzymes were chosen for their location on the main pathways for carbohydrate metabolism; the last one (esterases) for the great polymorphism they showed in a lot of microorganisms.

4. Polyacrylamide gel electrophoresis:

Protein samples were submitted to vertical electrophoresis on a 1.5 mm thick, 7.5% non denaturing polyacrylamid gel overlaid with a 4.5% polyacrylamide stacking gel using the discontinous buffer system of Laemmli (1970).

The concentrations of protein preparations were adjusted for each enzyme tested (50 to 200 µg/100µl) in order to obtain the best resolution and equal volumes of sample buffer (30µl) were loaded into each lane of the gel.

Electrophoresis was carried out at 10°C with a constant 15 mA current per slab until the tracking dye (bromophenol blue) reached the bottom of the gel.

5. Staining procedures of enzymes:

After electrophoresis, the gel slabs were placed directly into substrate solutions for the revelation of enzymatic activity. Histochemical reactions on the polyacrylamide gel were similar to those described in Pasteur et al. (1987).

6. Numerical analysis:

The relative electrophoretic mobilities (Rm) of the enzyme bands were calculated as the ratio of the distance-migration of the enzyme from the origin to that of the tracking dye.

The different patterns (i.e. electromorphs) observed for each enzyme were numbered from decreasing anodal mobility, and the electromorph diversity (H) estimated by the H coefficient. ($H = 1- Sx_i^2$, where i is the frequency of the ith electromorph).

Jaccard similarity coefficients for all possible pairs of isolates were calculated according to Gardes et al. (1987) and the relatedness between the strains determinated by the UPGMA method - Unweighted Pairwise Grouping Mathematical Average - (Piung Li, 1981) that was then used to generate a dendrogram (Sneath & Sokal, 1973).

RESULTS

Zymograms of the twelwe *Saccharomyces cerevisiae* strains tested are illustrated in schematic diagrams (Fig. 1).

All strains exhibited activity for all the enzymes studied. The number of electromorphs observed per enzyme as well as the electromorph diversity are showed in Table 2.

The Rm of aldolase (ALD) ranged from 0.18 to 0.38. Strains D and E showed two isoenzymes bands and strain F eight. Nine distinct electromorphs were noted with a H diversity of 0.88.

The Rm of alcohol deshydrogenase (ADH) were distributed from 0.19 to 0.45. Two bands (strain I) to ten (strain G) were observed, leading to ten different electromorphs (H= 0.89).

Glucose-6-phosphate deshydrogenase (G6PD) showed four isoenzyme bands very narrowly ranged (Rm 0.27 to 0.30) and most of the strains exhibited only one band (0.28 or 0.30) common respectively to eight or seven strains. Six electromorphs were observed (H= 0.81).

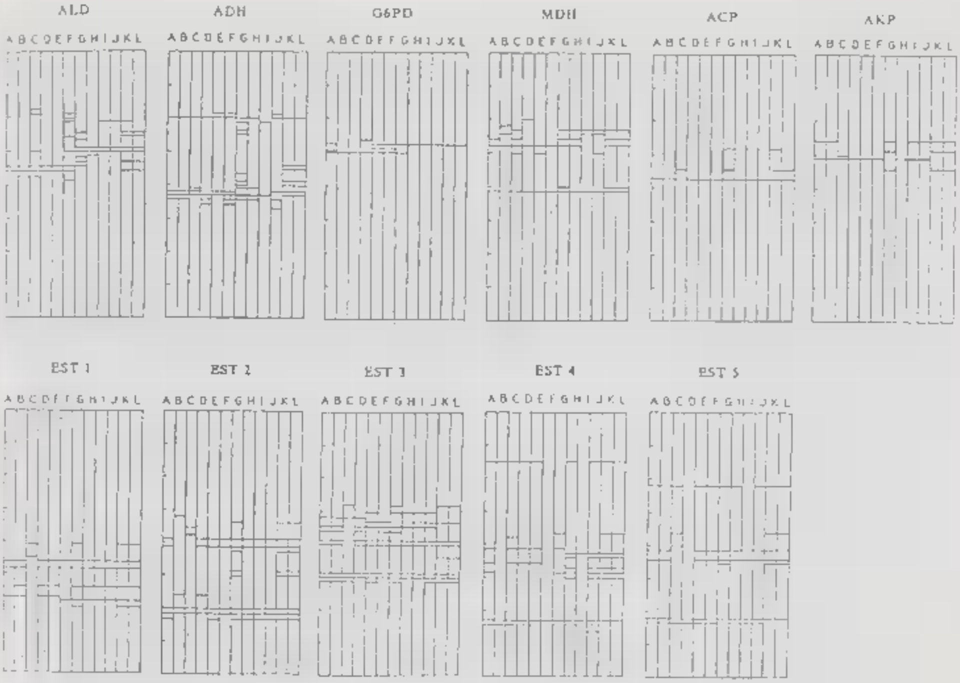

Figure 1: Diagram of the zymogram patterns of the 12 *Saccharomyces cerevisiae* strains studied.

Figure 1: Diagramme des profils enzymatiques des 12 souches de *Saccharomyces cerevisiae* étudiées.

Malate deshydrogenase (MDH) gave nine different bands ranged from Rm 0.20 to 0.41. Two bands (strain F) to five (strains C,G,K,L) were detected in one yeast leading to a great diversity of electromorphs (H= 0.87).

From all the enzymes tested, acid and alkaline phosphatases (ACP and AKP) exhibited the less high diversity with respectively three and four distinct electromorphs. Rm were distributed from 0.28 to 0.37 (ACP) and from 0.26 to 0.34 (AKP), no more than three different isoenzymes bands - and often only one - being detected in the strains.

Esterases showed the more anodal mobilities of all the enzymes studied. The Rm were widely ranged from 0.41 to 0.60 (EST 1), 0.32 to 0.63 (EST 2), 0.28 to 0.51 (EST 3), 0.15 to 0.65 (EST 4) and 0.22 to 0.63 (EST 5). The lowest diversity was observed for EST 2 (H= 0.74) and the highest for EST 4 with 10 distinct electromorphs (H= 0.89).

DISCUSSION

As shown in Figure 1 and Table 2, all strains of *Saccharomyces cerevisiae* tested exhibited a great diversity of electrophoretic isoenzymes banding patterns, thus suggesting a large strain heterogeneity. This diversity was clearly underlined for enzymes such as ALD, ADH, MDH or EST 3, 4, where eight to ten different electro-

Table 2: Electromorph diversity of enzymes.
Tableau 2: Diversité des profils électrophorétique des enzymes.

Strains	ALD	ADH	G6PD	MDH	ACP	AKP	EST1	EST2	EST3	EST4	EST5
A	2	4	2	6	1	4	4	1	1	6	4
B	2	4	4	9	1	4	4	6	1	10	4
C	3	6	4	3	2	3	5	5	5	9	6
D	4	2	5	10	1	2	4	2	7	8	1
E	4	10	1	2	1	2	4	1	2	8	4
F	1	3	1	5	1	2	3	4	6	2	4
G	9	7	3	1	3	1	1	3	4	7	3
H	7	5	6	7	1	2	2	1	8	4	4
I	8	8	6	8	1	4	2	1	8	3	2
J	8	1	6	4	1	4	2	1	2	1	4
K	6	9	6	1	3	1	1	3	3	5	5
L	5	9	6	1	2	1	1	3	3	5	5
n° of strains	12	12	12	12	12	12	12	12	12	12	12
electromorphs	9	10	6	10	3	4	5	6	8	10	6
H diversity	0,88	0,89	0,81	0,87	0,51	0.71	0.75	0,74	0,86	0,89	0,77

morphs were detected with high H diversity coefficients narrowly ranged (0.86 to 0.89). However, this suggested heterogeneity was less obvious with enzymatic systems such as ACP or AKP which exhibited only three or four distinct electromorphs and low H coefficients (0.51 and 0.71 respectively).

When examined on the basis of precise conventional taxonomic criteria (Martins et al. 1990; Barnett et al., 1991), the main difference between all these yeasts was the assimilation of galactose, positive in strains B, C, D, G, I, J and L and negative in strains A, E, F, H and K. So, according to Van Der Walt (1970) they could be assignated into two clusters, the first ones in a "*Saccharomyces cerevisiae*" sub-group, and the last ones in a "*Saccharomyces bayanus*" sub-group.

To determine further the extend of relatedness between all the strains studied, the triangular matrix of Jaccard similarity coefficients was established (Table 3). The strains, all together, exhibited a relatively low similarity coefficient (0.14), the highest -0.82- being K/L ("*S. bayanus*" / "*S. cerevisiae*"), and the lowest -0- ("*S. cerevisiae*" strain C/ all other strains, B excepted). The so-called "*S. bayanus*" strains were found related to 0.19 average, with a range of distribution from 0 (K/A,E, F) to 0.36 (E/F). The so-called "*S. cerevisiae*" strains were also divergent with an average similarity coefficient of 0.09, with values from 0 (C/ other "*S. cerevisiae*" strains, B excepted) to 0.54 (I/J).

Indeed, "*S. cerevisiae*" and "*S. bayanus*" strains were widely overlaping and our results agreed quite well with numerical analysis -85 to 90% similarity between these two yeasts- (Campbell, 1972), cell wall antigenic structure -antigens 1, 10, 18, 2, 3, 14, 31 -(Tsuchiya et al., 1974), G + C content -38.8 to 40.2- (Nakase & Komagata, 1971) or pulsed field electrophoresis (Kaneko & Banno, 1991) and confirmed conclusions of Yarrow (1984) who considered *S. cerevisiae* and *S. bayanus* as synonyms.

Referring to the triangular matrix, similarity average values were calculated by the UPGMA method, and strains were included in the resulting dendrogram (Fig. 2).

Table 3: Matrix of pairwise Jaccard similarity coefficient between strains.
Tableau 3: Matrice des coefficients de similarité inter-souches (coefficients de Jaccard)

Strains	A	B	C	D	E	F	G	H	I	J	K	L
A	1,00											
B	0,64	1,00										
C	0,00	0,09	1,00									
[illegible]	0,18	0,18	0,00	1,00								
E	0,36	0,27	0,00	0,36	1,00							
F	0,18	0,18	0,00	0,18	0,36	1,00						
G	0,00	0,00	0,00	0,00	0,00	0,00	1,00					
H	0,27	0,18	0,00	0,18	0,36	0,27	0,00	1,00				
I	0,27	0,18	0,00	0,09	0,18	0,09	0,00	0,45	1,00			
J	0,36	0,27	0,00	0,09	0,36	0,18	0,00	0,36	0,54	1,00		
[illegible]	0,00	0,00	0,00	0,00	0,00	0,00	0,45	0,09	0,09	0,09	1,00	
[illegible]	0,00	0,00	0,00	0,00	0,00	0,00	0,36	0,09	0,09	0,09	0,82	1,00

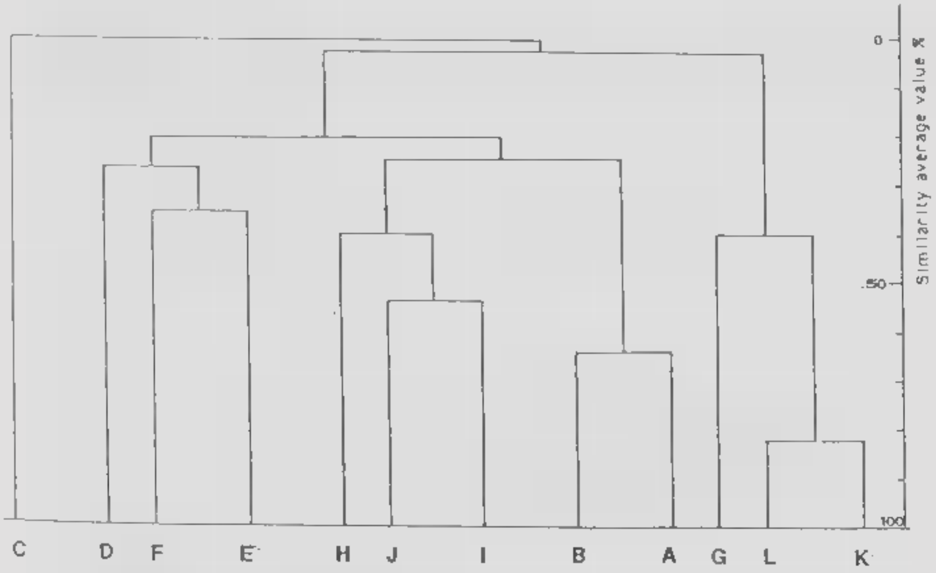

Figure 2: Dendrogram of 12 *Saccharomyces cerevisiae* strains studied on the basis of similarity average values (UPGMA method).

Figure 2: Dendrogramme des 12 souches de *Saccharomyces cerevisiae*, établi à partir des coefficients de similarité (calcul UPGMA).

With the exception of strain C, all other strains could be assignated into two clusters, the first one including strains G, L and K and the second one the remaining yeasts. In the first cluster, strains K ("*S. bayanus*") and L ("*S.* cerevisiae") revealed a high similarity value (82%) and were related to strain G ("*S. cerevisiae*") at 40% matching coefficient. In the second cluster, strain A ("*S. bayanus*") and B ("*S. cerevisiae*") were the most closely related (64%). Strains I and J ("*S. cerevisiae*") matched at 54% and were both related at 40% with strain H ("*S. bayanus*"). Strains E and F ("*S. bayanus*") -36% of similarity average value- were related to strain D ("*S. cerevisiae*") at 27%; it was approximately the same -25.5%- for A, B/ H, I, J, while all the strains here included matched at 21%. No relationship closer than 3% matching was noted between these two clusters, while strain C ("*S. cerevisiae*") matched no more 1% with both them. So, numerical analysis clearly showed mutually wide matching coefficient between all these yeasts without allowing an obvious discrimination into two separate taxa.

However, when examined for deoxyribonucleic acid relatedness, *S. cerevisiae* and *S. bayanus* showed non substantial DNA homology -5% (Vaughan-Martini & Kurtzman, 1985; Vaughan Martini & Martini, 1987, Kaneko & Banno, 1991). Further, Kurtzman & Robnett (1991) reported distinct partial sequences of 18S and 25S ribosomal RNA for these two species. So, even if some differences can be shown among these yeasts, all the strains here investigated must be considered merely as physiological variants of a same species, *S. cerevisiae*.

CONCLUSION

As an evidence, yeast classification mainly based on phenotypic characters such as morphological and physiological properties or biochemical reactions is not always satisfactory, especially in the delineation of closely related organisms. Because of strain variability in standard hydrocarbon assimilation and fermentation tests, the *Saccharomyces sensu stricto* species offer a good example of this problem. Molecular biology techniques such as electrophoretic comparison of enzymes or total soluble cell proteins as well as nuclear DNA reassociations, restriction patterns of mitochondrial DNA or sequences of larger ribosomal RNA may provided increased informations to assess more acurately relatedness among species.

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